Modulation by protein kinase C activation of rat brain delayed-rectifier K^+ channel expressed in *Xenopus* oocytes

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Abstract The modulation by protein kinase C (PKC) of the RCK1 K^+ channel was investigated in *Xenopus* oocytes by integration of two-electrode voltage clamp, site-directed mutagenesis and SDS-PAGE analysis techniques. Upon application of β-phorbol 12-myristate 13-acetate (PMA) the current was inhibited by 50–90%. No changes in the voltage sensitivity of the channel, changes in membrane surface area or selective elimination of RCK1 protein from the plasma membrane could be detected. The inhibition was mimicked by 1-oleoyl-2-acetyl-rac-glycerol (OAG) but not by αPMA, and was blocked by staurosporine and calphostin C. Upon deletion of most of the N-terminus a preceding enhancement of about 40% of the current was prominent in response to PKC activation. Its physiological significance is discussed. The N-terminus deletion eliminated 50% of the inhibition. However, phosphorylation of none of the ten classical PKC phosphorylation sites on the channel molecule could account, by itself or in combination with others, for the inhibition. Thus, our results show that PKC activation can modulate the channel conductance in a bimodal fashion. The N-terminus is involved in the inhibition, however, not via its direct phosphorylation.

Key words: K^+ channel; Protein kinase C; Xenopus oocyte; Phosphorylation; Biphasic modulation; Site-directed mutagenesis

1. Introduction

Voltage-gated K^+ channels are a diverse group of ion channels present in most eukaryotic cells. A major function of these channels is to set the membrane potential and hence regulate the electrical excitability of the cell [1].

Regulation of channel function by protein phosphorylation has been described for a number of different classes of K^+ channels in their native cellular environment [2]. The use of *Xenopus* oocytes enhanced understanding of the regulation by phosphorylation of K^+ channels expressed from a single species of cRNA. Thus, for example, we were able to show that the A-type channels expressed from either chick brain RNA [3] or Shaker H4 cRNA [4] are modulated by PKC activation. Moreover, the use of *Xenopus* oocytes allows correlation of protein kinase-mediated modulation with direct phosphorylation of channels by integrating electrophysiological analysis with molecular biology techniques. Thus, it was shown that the function of a slowly activating K^+ channel (minK) is modulated by PKC activation through phosphorylation of a serine residue on the channel molecule [5] and by PKA activation, but not through direct channel phosphorylation [6,7]. The Shaker D, Shaker B [8] and Kv1.2 [9] channels were shown to be modulated by PKA activation via phosphorylation of a specific serine residue in each molecule and the hKv3.4 channel was shown to be modulated by PKC phosphorylation of two serine residues [10].

Recently, with the addition of a biochemical approach to the study of K^+ channels in oocytes, we could show that the rat brain RCK1 channel (Kv1.1; ref. [11]), that functions in oocytes as a delayed rectifier-type of K^+ channel [12], is modulated by PKA activation through two separate mechanisms, one via direct channel phosphorylation at Ser-446 on the C-terminus and the other involving enhanced synthesis of the channel protein [13,14]. In the present work we have extended the study of this channel and characterize its modulation by PKC activation. We also show that, in contrast to the PKA modulation, the N-terminus is probably involved in the PKC modulation and not through direct channel phosphorylation.

2. Materials and methods

2.1. Site-directed mutagenesis

All mutations were performed on the expression vector SupEx-RCK1, which confers high levels of expression [13]. Oligonucleotide-mediated mutagenesis was performed using the Amersham mutagenesis kit, essentially as described [13], using single-stranded DNA as templates and oligonucleotide primers (synthesized by General Biotechnology Inc., Rehovot) encoding the desired mutations. Subsequent mutations were prepared using a template derived from the wild type (WT) DNA and the following primers (bold nucleotides encode the mutated residues): S322C, 5′ CCTCAAAGC**TTGT**ATGAGGGA 3′;R324M, 5′ GCTAGTATG**ATG**GAGTTAGGG 3′;S489I, 5′ GTTAATAAG**ATC**AAGCTCCTG 3′;R443C;S446A, 5′ GTGACCTCAG**CTGC**CGCAGC**GCC**TCTACTATCAGC 3′;S23I, 5′ CAGGATGGC**ATC**TACCCAAGG 3′;R47G;K53Q, 5′ CTCCGGGCTG**GGC**TTCGAGACGCAGCTC**CAG**ACTCTGGC 3′;S88I, 5′ AACCGGCCC**ATC**TTCGATGCC 3′;S98A, 5′ TTACTACCAG**GCG**GGGGGGGCG 3′.

The mutations S332C;S489I and S322C;R47G;K53Q were prepared using the template derived from S322C DNA and the appropriate primers (see above). The mutation S322C;R47G;K53Q;S98A was prepared using S322C;R47G;K53Q as a template and the appropriate primer. The del-N construct was prepared by digestion of the SupEx-RCK1 with *Nco*I (deletion of the first 126 amino acids from the N terminus). The construct del-N with S322C;S489I was prepared by digestion of S332C;S489A with *Nco*I. All mutations cDNAs were linearized by *Not*I and the corresponding cRNAs synthesized by T7 RNA polymerase.

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2.2. Oocytes, electrophysiological recording, drug application and statistics

Frogs were maintained and dissected and oocytes were prepared as described [13]. The oocytes were injected with about 0.2 ng cRNA and incubated at 22°C for 3–6 days in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5) supplemented with 1.8 mM $CaCl_2$, 2.5 mM sodium pyruvate, 100 μg/ml streptomycin and 100 U/ml penicillin (NDE solution), and then assayed electrophysiologically. An oocyte was placed in a 1 ml bath constantly perfused with the ND96 solution supplemented with 1 mM $CaCl_2$. Currents were recorded using a Dagan 8500 two-electrode voltage clamp amplifier with a series resistance compensation circuit and low resistance (0.2–0.5 MΩ) electrodes [14]. Data acquisition and analysis were done by the pCLAMP software (Axon Instruments, Foster City, CA, USA; ref. [14]). The holding potential was set to −80 mV and the potential was then stepped to 0 mV for 150 to 400 ms. The net current was estimated by arithmetical subtraction of the scaled leak current calculated from the current elicited by a voltage step to −90 mV. When current/voltage relationship was studied, the holding potential was set at −80 mV and stepped to −100 mV for 50 ms (prepulse), before depolarizing the membrane potential to different voltages for 150 ms. The net current was estimated by subtraction of the leak current that was the average value of scaled currents elicited by voltage steps to −80 and −70 mV. The capacitance of the oocyte membrane was measured as the area under the capacitive current elicited by a step from −80 to −70 mV. After stability of the current

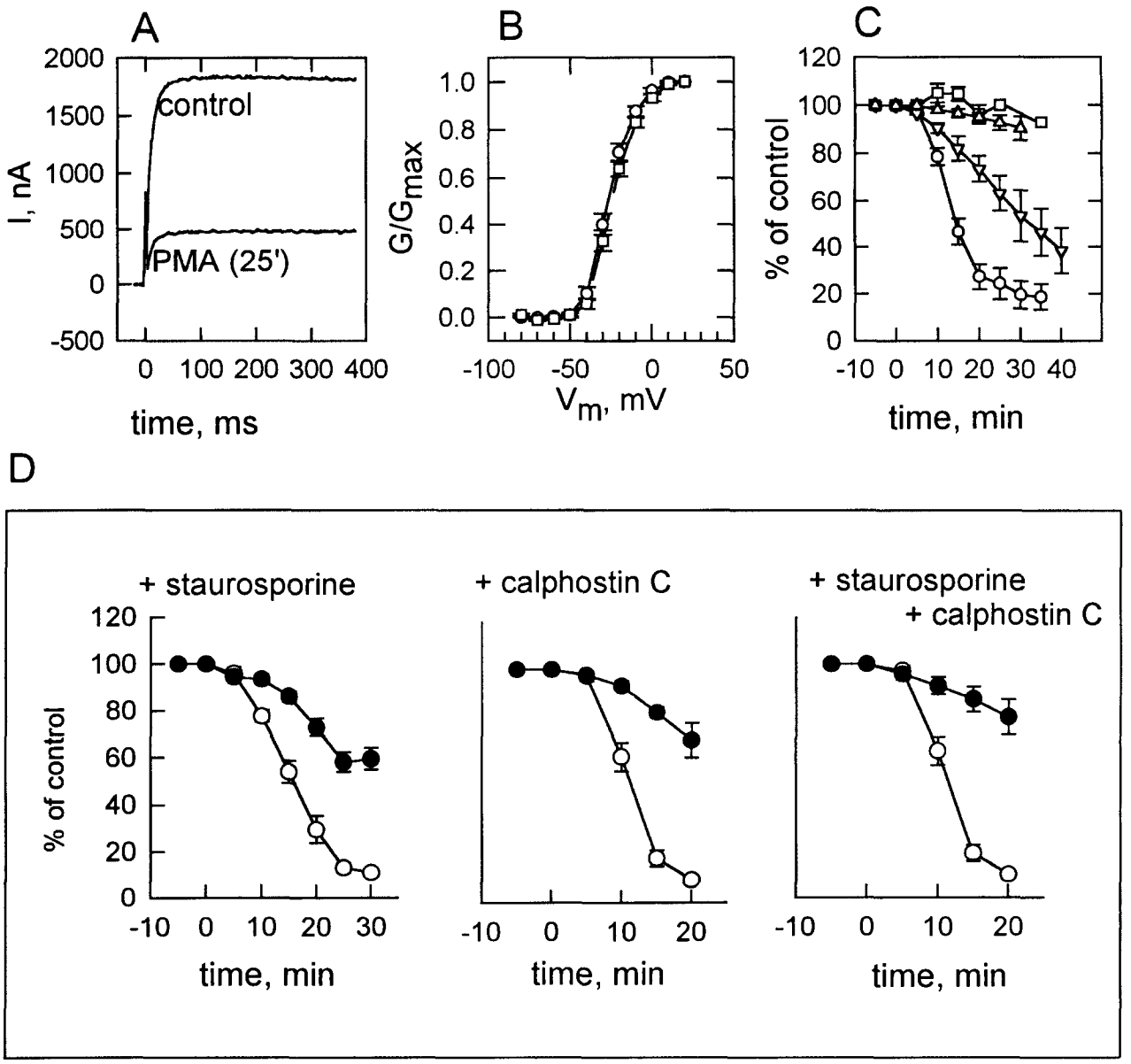

Fig. 1. Modulation of I_{RCK1} by PKC activation. A: Currents elicited by voltage steps from a holding potential of −80 mV to 0 mV before (control) and 25 min after PMA application. B: Steady-state activation curves of I_{RCK1} before (circles, $n=9$) and 20 min after (squares, $n=6$) application of 10 nM PMA. A 50 ms prepulse to −100 mV was applied, followed by a test pulses to various voltages (−80 to +20 mV) in 10 mV increments, every 4 s (at +20 mV maximal conductance was observed). C: Time scale of the effects on I_{RCK1} of 10 nM PMA (circles, $n=15$), 10 mM OAG (downward triangles, $n=5$) and 100 mM αPMA (upward triangles, $n=5$). Squares represent the effect of 10 nM PMA on capacitance (n = 7; see section 2). D: Effects of PKC inhibitors. The effects of PMA on I_{RCK1} in oocytes (filled symbols) that were previously exposed for 0.5–2 h to 3 μM staurosporine (left panel, $n=6$), for 1–6 h to 5 μM calphostin C (middle panel, $n=3$), and for 1–6 h to both inhibitors (right panel, $n=3$). Untreated oocytes are represented by open symbols ($n=4$; 3; 3, respectively). In C and D, the currents were elicited by 400 ms voltage steps from −80 mV to 0 mV. Shown are the mean normalized current amplitudes with error bars that represent S.E.M. In all cases net currents are shown (see section 2).

amplitude had been verified for at least 5 min (usually up to 15 min), β-phorbol 12-myristate 13-acetate (βPMA), αPMA, and diacylglycerol 1-oleyl-2-acetyl-rac-glycerol (OAG) were applied by bath perfusion. Inhibition by staurosporine was assessed in oocytes incubated in 3 μM solution for 0.5 to 2 h, prior to drug application. Inhibition by calphostin C was assessed in oocytes incubated in 5 μM solution for 1 to 6 h (illuminated under standard fluorescent light). The results in the text are presented as means ± S.E.M. Two-tailed *t*-test was used to calculate the statistical significance of the differences between two populations.

2.3. Immunoprecipitation

Labeling of oocytes, plasma membranes preparation, homogenization and immunoprecipitation were done as described [13,14]. Quantification of labeling intensities and generation of digitized PhosphorImager (Molecular Dynamics) scans were done essentially as described [14].

2.4. Materials

All substances were purchased from Sigma except αPMA (LC-Services; Woburn, MA) and OAG (Calbiochem). αPMA, βPMA, OAG and staurosporine were dissolved at 10 mM in DMSO and kept at −20°C. PMA and OAG were light protected, OAG was also stored under nitrogen. Final concentrations were made up in ND96 solution just before use.

3. Results

3.1. Modulation of the full-length RCK1 channel

About 3 to 6 days after injection of cRNA coding for the RCK1 rat brain voltage-gated K^+ channel into *Xenopus* oocytes, the K^+ current (I_{RCK1}) was recorded using the two-electrode voltage clamp technique. As reported previously [12], the current was of a delayed-rectifier-type (Fig. 1A). The modulation of I_{RCK1} by PKC was examined first by constant bath perfusion with the PKC activator, PMA. Within 5 to 10 min the current amplitude started to decline and by 20 min had reached about 27% of its original value ($P<0.001$; Fig. 1A,C). Three types of control experiments were carried out to verify that the effect of PMA was indeed due to its activation of PKC: (i) αPMA (10 nM), an isomer that is inactive with respect to PKC, had practically no effect (Fig. 1C). (ii) The synthetic diacylglycerol OAG (10 μM), another activator of PKC, reduced I_{RCK1} amplitude to 40% of its original value after 40 min ($P<0.001$; Fig. 1C). (iii) Two protein kinase inhibitors, staurosporine and calphostin C [15,16] attenuated the effect of PMA (Fig. 1D). To overcome possible variability in the PMA effect among different donors, we compared in the same donor the effects of PMA in the presence and absence of the inhibitors. Thus, the effects of PMA were weakened by staurosporine and by calphostin C by about 60% ($P<0.001$) and 85% ($P<0.001$), respectively. Simultaneous application of the two inhibitors (calphostin C interacts with the regulatory domain of PKC while staurosporine interacts with the catalytic domain) weakened the effect of PMA by 87% ($P<0.00$). Staurosporine alone had practically no effect as it decreased I_{RCK1} amplitude to 96.2 ± 2.8% ($n=5$) of control after 20 min.

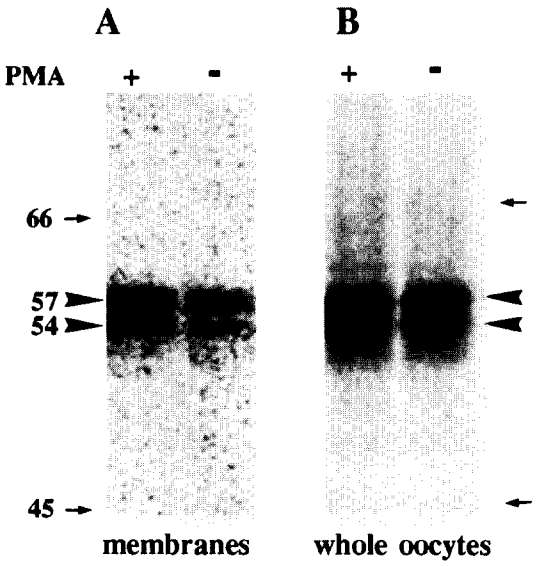

Fig. 2. PMA treatment does not affect the amount of RCK1 protein in plasma membranes (A) and in whole oocytes (B). Shown are digitized PhosphorImager scans of SDS-PAGE analysis of [^{35}S]met/cys-labeled RCK1 protein products immunopurified from either 17 plasma membranes or five whole oocytes after incubation for 20 min in the presence and absence of 100 nM PMA (as indicated). The scans in A and B were derived after 5 and 1 days of exposure, respectively. Thin arrows denote molecular weight markers in kDa.

To assess the effect of PMA on the voltage sensitivity of the channel steady-state activation curves of currents before and during PMA application were compared. Groups of currents evoked by depolarizing steps to various voltages before and 20 min after PMA application were recorded. Conductances (G) at all voltages were calculated and normalized to peak conductance at +20 mV (G_{max}) and steady-state activation curves were derived (Fig. 1B). The two curves practically overlapped, indicating that the effect of PMA was not the result of a positive shift in the voltage dependency of activation.

In order to exclude the possibility that the effect of PMA is to accelerate internalization of the channels rather than to affect the channel function, we carried out two sets of experiments. Monitoring the capacitance of the oocyte membrane during the PMA application revealed that it did not change significantly during the experiment (Fig. 1C), indicating that the decrease in I_{RCK1} was not due to a decrease in the membrane surface area. Furthermore, SDS-PAGE analysis of the immunopurified RCK1 protein (that is expressed in the oocyte in the form of a doublet; see refs. [13,14]) revealed that PMA treatment did not affect the amount of total or the plasma membrane protein content of the oocytes. Thus, the estimated ratios of the amount of protein in PMA treated (10 or 100 nM for 20 or 60 min) versus untreated oocytes were 1.1 ± 0.1 and 1.14 ± 0.19 (15 and 4 experiments, respectively) for the total and plasma membrane proteins, respectively (see Fig. 2). It was therefore concluded that the PMA-induced decrease in I_{RCK1} was not due to a reduction in the amount of plasma membrane RCK1 channels that were shown to represent functional channels [13].

3.2. Modulation of the N-terminus truncated channel

Deletion of the first 126 amino acids from the N-terminus generated a functional channel (del-N); however, this channel required cRNA concentrations two orders of magnitude larger than those in the full-length channel for expressing comparable current amplitudes. The kinetics of the current through del-N (I_{del-N}) were similar to those of I_{RCK1} (compare Figs. 1A and 3B) and its voltage sensitivity did not seem to be altered (Fig. 3A). However, 10 nM PMA caused a biphasic response of I_{Del-N} with an early increase of current to about 140% of the initial value ($P<0.003$), which peaked after about

6 min, followed by a significant reduction of the current to about 30% of the initial value ($P<0.0005$), within 25 min. The extent of reduction was about 50% of the corresponding reduction in I_{RCK1} in oocytes of the same donors ($P<0.003$; Fig. 3C). OAG (10 μM) mimicked both the enhancement ($P<0.0025$) and the suppression ($P<0.0005$) of the current amplitudes which were of comparable magnitude to those induced by PMA (tested in the same donors; Fig. 3E). Both effects induced by PMA were significantly attenuated in the presence of 3 mM staurosporine (at 7 min $P<0.05$; at 25 min $P<0.001$; Fig. 3E). The steady-state activation behaviors of I_{del-N} at 6 and 20 min after PMA application were practically unaffected (Fig. 3D), indicating that both the suppression and the initial enhancement of I_{del-N} occurred not because of altered voltage sensitivity but probably because of changes in the conductance. Also, the kinetics of I_{del-N} did not seem to be altered by PMA (Fig. 3B).

In view of the early enhancement of the PMA effect on

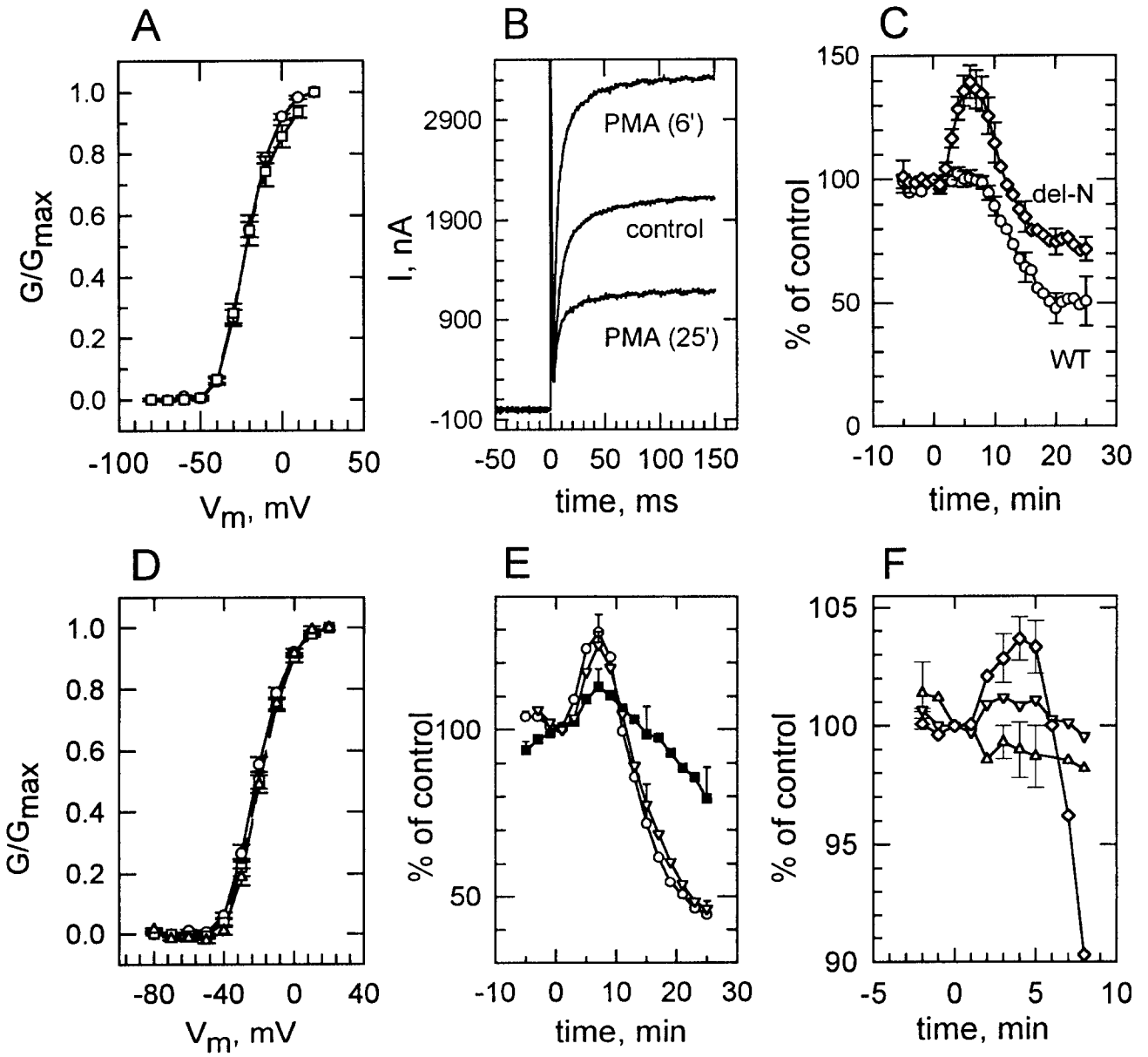

Fig. 3. Modulation of I_{del-N} by PKC activation. A: Steady-state activation curves of I_{RCK1} (squares, $n=4$) and I_{del-N} (circles, $n=5$). A 50 ms prepulse to −100 mV was applied, followed by test pulses to various voltages (−80 to +20 mV) in 10 mV increments (at +20 mV maximal conductance was observed). B: I_{del-N} elicited by 150 ms pulses from a holding potential of −80 mV to 0 mV, before (control), 6 min after (6') and 25 min (25') after PMA application. C: Time scale of the effect of 10 nM PMA on I_{RCK1} (circles, $n=8$) and on I_{del-N} (diamonds, $n=9$). Shown are mean normalized currents with error bars that represent S.E.M. D: Steady-state activation curve of I_{del-N} ($n=6$) before (circles), 6 min (squares) and 15 min (triangles) after PMA application. A 50 ms prepulse to −100 mV was applied, followed by test pulses to various voltages (−80 to +20 mV) in 10 mV increments. E: Time scale of the effects of 10 nM PMA (open circles, $n=11$), 10 mM OAG (open triangles, $n=3$) and 10 nM PMA after exposure for 0.5–2 h to 3 mM staurosporine (squares); note different Y axis scale. Shown are mean normalized currents with error bars that represent S.E.M. F: Enlargement of the figure showing the I_{RCK1} response to PMA in the first minutes after the drug application. Time course of the effects of vehicle wash (upward triangles), 1 nM PMA (downward triangles), 10 nM PMA (diamonds). The figure shows the mean normalized currents with error bars that represent S.E.M., where the augmentation is significantly different from the vehicle wash values; $n=5$ for all treatments.

I_{del-N}, we carefully re-examined the effect of PMA on I_{RCK1}. In oocytes of most of the donors tested we found an early tiny increase of the current amplitude that was reproducible and statistically significant at 10 nM PMA (4%; $P<0.05$; Fig. 3F).

3.3. The role of 'classical' consensus PKC sites in the PMA-induced effects

Ten PKC phosphorylation sites, serine or threonine flanked by basic amino acids [17], were identified on the assumed intracellular parts of the RCK1 channel protein (see Fig. 4). Mutant cDNAs were generated in which one or more of these sites were truncated by replacement of either the serine, threonine or the basic amino acids with different amino acids (see section 2). Since the expression of currents is sometimes impaired by mutations, we ensured their maximum expression by using a construct of the channel in a 'super-expressing' vector that confers high levels of expression in oocytes (see section 2). The corresponding cRNAs were injected into the oocytes and the responses to PMA of the currents through the resulting mutant channels were examined. None of the mutations affected significantly the responses of the currents to PMA. In an attempt to probe for synergistic interactions among several potentially phosphorylation sites, several simultaneous mutations at different parts of one RCK1 molecule were generated. Simultaneous mutations in the full length channel at Ser-322, Ser-44, Thr-49 Thr-54 and Ser-98, as well as simultaneous mutations at Ser-322 and Ser-489, did not affect the PMA-induced modulation of the current. Simulataneous mutations in the del-N construct at Ser-322 and Ser-489 resulted in a channel that did not express current.

4. Discussion

4.1. Bimodal effect of PKC activation

In this study we show that RCK1 channel expressed in oocytes is modulated by PKC activation. The dominant effect appears to be a strong inhibition of 50% to 90% (depending on the donor). Examination of the mechanism underlying the inhibition revealed that it is not due to a shift of the voltage sensitivity to more positive potentials, nor does it result from non-specific internalization of membrane proteins due to overall reduction in membrane surface area. Moreover, by comparing the amounts of RCK1 protein in the plasma membranes of treated and untreated oocytes, we present evidence that the inhibition is not due to a selective elimination of K^+ channel proteins from the plasma membrane. We therefore conclude that the inhibition results from reduced RCK1 conductance. The nature of this reduced conductance, in particular whether it is manifested in the intrinsic characteristics of single channels or involves other mechanisms, e.g. recruitment of channels to non-functional pools (see also ref. [14]), remains to be determined.

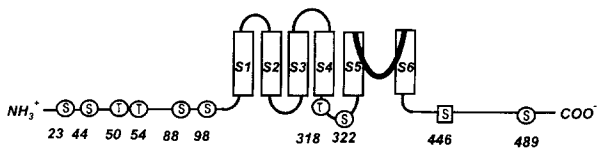

Fig. 4. Scheme of the proposed transmembrane topology of the RCK1 protein (monomer). The PKC consensus phosphorylation sites that were truncated in this work are denoted by S (serine) and T (threonine). Serine 446 (in square) is the site of PKA phosphorylation [13].

Interestingly, upon truncation of the N-terminus, an early-phase enhancement of the current amplitude of about 40% is observed. Like the inhibition, the enhancement is a result of PKC activation, and probably occurs via changes in the channel conductance. Such effects of a bimodal nature by PMA have been described for other voltage-gated ion channels. We [18] and others [19] have described such effects for L-type Ca^{2+} channels either expressed in *Xenopus* oocytes or derived from ventricular myocytes [20,21], and for the human Kv1.1 K^+ channel expressed in *Xenopus* oocytes [22]. When concidering the mechanism underlying the bimodal nature of the PMA-induced effect on the truncated RCK1 channel one possibility arises that the enhancement is a component of the full length channel modulation that is obscured by the overlapping inhibition and is revealed when 50% of the inhibition is removed upon N-terminus deletion. In such a context the observed initial tiny enhancement of the current through the full-length channel represents the underlying enhancement. Another possibility is that the enhancement is a novel phenomenon introduced by the deletion of the N-terminus. This requires that the truncation renders new sites on the molecule responsive to PKC activation, sites that are not available in the full-length channel because of an unfavorable tertiary and/or quaternary conformation imposed by the N-terminus. Indeed it seems reasonable to assume that the structure of the Kv1.1 molecule is influenced by the N-terminus, which participates in the assembly and contributes to the stabilization of the multimeric channel [23]. Supportive data come from the study of Stocker et al. [24] that demonstrated that two alternatively spliced *Drosophila* Shaker K^+ channels, differing only in their amino termini, exhibited profound differences in their sensitivities towards tetraethylammonium and charybdotoxin that interact with the pore region of the molecule. Also, Wilson et al. [25] have shown that the drk1 K^+ current can be modulated by protein kinase A only upon deletion of the first 139 amino acids of the N-terminus.

The question arises whether the enhancement effect that becomes significant only after truncation of the N-terminus is of relevance to the physiological functioning of the full-length channel. Notably, two Kv1.4 homologs which are 95.6% identical in their amino acid sequence do not respond similarly to PMA, when expressed in *Xenopus* oocytes. The rat homolog undergoes only suppression over time with no initial enhancement observed [26], whereas the human homolog responds in a biphasic manner with an initial enhancement followed by suppression [22]. Most of the differences (76% mismatch) between these two molecules lie in the N terminus suggesting that alterations in the N-terminus may regulate the responses to intracellular signals, such as PKC activation. Interpretation of the RCK1 modulation data leads us to further speculate that such regulation of reponses to PKC activation may be achieved even with the same channel molecule. One can assume that in the full-length channel engagement of the N-terminus by interactions with intracellular protein(s) has the same consequences on the channel structure as N-terminus deletion, hence affecting the modulation of the channel by PKC activation.

4.2. *The inhibitory effect is mediated partially by the N-terminus, probably not via direct phosphorylation*

Upon removal of most of the N-terminus about 50% of the inhibition induced by PKC activation was eliminated suggesting that this part of the molecule is responsible for a significant portion of the inhibitory effect. The question arised whether this part of the inhibition, as well as the rest of the inhibition, are mediated via direct phopshorylation of serine or threonine residues on the channel molecule. Several membrane proteins have been shown to be modulated by PKC activation via direct protein phosphorylation. These include the rat kidney minK [5] and the human hKv3.4 [10] K^+ channels, the Na^+ channel [27,28] the $GABA_A$ receptor [29,30], and the Na,K-ATPase α subunit [31]. We found that this is probably not the case with the RCK1 channel as phosphorylation of none of the classical potential PKC phosphorylation sites [17] on the RCK1 protein (six of which reside in the N-terminus) could account for the inhibitory effect by the PKC activation. Also, no synergistic effect among the phosphorylation sites such as had been observed in the case of CFTR [32] and the Na^+ channels [33] could be detected. Nevertheless, PKC-recognition sequences are diverse, and a broad range of substrate specificity has been reported [34]. Also, a novel motif for PKC-mediated phosphorylation was recently identified for the Na,K-ATPase molecule which is not contained within a conventional consensus sequence for PKC [31]. Therefore, we can not rule out completely the possibility that the inhibitory effect by PKC could be conveyed through direct channel phosphorylation of sites not yet identified. The possible involvement of tyrosine phopshorylation in this modulation has been also considered in view of the recent data describing the modulation of a related voltage-gated K^+ channel [36,37]. This possibility, however, does not seem to us plausible as a tyrosine kinase inhibitor (genistein) did not affect the modulation by PKC (data not shown).

Activation of PKC has been implicated in plasticity phenomena in the brain, such as long-term potentiation, although the underlying mechanisms are not fully understood (for review see ref. [35]). One of such mechanisms may be a long-lasting inhibition by PKC of a voltage-dependent K^+ channel, such as described in this report, which may be expected to cause a prolonged increase in nerve cell excitability.

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